Autosomal Albino Chicken Mutation (c^a/c^a) Deletes Hexanucleotide (−ΔGACTGG817) at a Copper-Binding Site of the Tyrosinase Gene[1]

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ABSTRACT We compared tyrosinase cDNA sequences from a line of autosomal albino and Black Silky chickens isolated from cultured melanocytes by reverse transcription-polymerase chain reaction (RT-PCR). Both sources produce a single DNA fragment of predicted normal tyrosinase size. Direct sequencing of the PCR product showed three mutated sites in the tyrosinase gene of the albino chicken. Two silent point mutations and a deletion of six nucleotides (−ΔGACTGG) at 817 bp in the tyrosinase cDNA sequence were observed when compared with the White Leghorn and Black Silky cDNA sequences. The deduced albino chicken tyrosinase protein lacks two amino acids, aspartic acid and tryptophan. The position of these amino acids is consistent with one of the potential copper-binding sites that should be indispensable for function of the enzyme. We speculate that the six-base deletion is responsible for the inactive tyrosinase in this line of albino chickens.

(*Key words*: chicken, albino, mutation, tyrosinase, copper-binding site)

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INTRODUCTION

Melanin is a pigment producing brown or black color in the retina, iris, skin, hair, and feathers of a wide range of vertebrates. Melanin synthesis occurs in pigment cells, or melanocytes, of the pigment epithelium, iris, integument, and feather follicles of birds. The first biochemical step is known to be the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) followed by the dehydrogenation of DOPA to dopaquinone (Lerner and Fitzpatrick, 1950). In the melanin biosynthetic pathway, tyrosinase is the key enzyme involved in these first two reactions as well as in the dehydrogenation of 5,6-dihydroxyindole-2-carbonic acid (DHICA) in a subsequent reaction (Korner and Pawelek, 1982).

Albinism has been reported in almost all vertebrates and is due to a defect in melanin synthesis arising from genetic mutations. The degree of depigmentation caused by different albino mutations varies from complete to incomplete. Most examples of complete albinism in higher vertebrates have been due to defects in the tyrosinase gene. In the case of human and mouse, the *C* locus has been genetically defined as the structural tyrosinase gene locus.

Three types of albinism at the *C* locus have been reported in the chicken, e.g., red-eye white (c^{re}), recessive white (c), and autosomal albino (c^a) (Brumbaugh *et al.*, 1983). Thus, the chicken *C* locus is a multiple allelic locus. According to Smyth *et al.* (1990), the most dominant allele is wild type (C^+), with strong pigmentation, and the most recessive is autosomal albino (c^a) showing complete lack of pigmentation. An earlier investigation using two-dimensional gel electrophoresis by Oetting *et al.* (1985) suggested that the *C* locus of the fowl is the structural locus for tyrosinase. Additionally, Boissy *et al.* (1987) have reported that the c^a mutation produced a protein that is functionally and antigenically different from normal tyrosinase. Brumbaugh *et al.* (1983) used the normal mouse tyrosinase cDNA to perform *in vitro* gene therapy in c^a/c^a melanocytes. Pigmented melanocytes are produced when albino melanocytes are infected with an avian leukosis virus vector containing the normal mouse tyrosinase cDNA (Whitaker *et al.*, 1989; Frew *et al.*, 1992; Akiyama *et al.*, 1994). These preceding reports strongly suggest that the c^a/c^a albino chicken has a mutation in the tyrosinase

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Abbreviation Key: RT-PCR = reverse transcription-polymerase chain reaction; aa = amino acid; DOPA = dihydroxyphenylalanine; DHICA = 5,6-dihydroxyindole-2-carboxylic acid; ORF = open reading frame.

gene. However, there has been no confirming molecular information.

In this study, we performed molecular analysis of the tyrosinase gene in the albino chicken (c^a/c^a) by using RT-PCR and direct sequencing. Here we report a six-nucleotide deletion in the tyrosinase cDNA of the albino chicken and discuss how the mutation may affect enzymatic function.

MATERIALS AND METHODS

Animals

A chicken albino line c^a/c^a (Brumbaugh *et al.*, 1983) has been transferred from the USDA-Agricultural Research Service Avian Disease and Oncology Laboratory. A homozygous albino line was reisolated after crossing with Nagoya Cochin, and has been maintained inbred at the Aichi-ken Agricultural Research Center. This albino line was considered to carry the tyrosinase (c) allele mutation as Brumbaugh's original c^a/c^a because the albinism in this line was rescued in the same way as the original c^a/c^a by transfection with the mouse tyrosinase gene (Akiyama *et al.*, 1994). A strain of heavily pigmented chickens, the Black Silky, has been maintained in the same center. Three-day embryos of albino and Black Silky chickens were used for cell isolation and 3-, 6-, and 9-d embryos were used for tissue (muscle, brain, and liver) isolation to prepare tyrosinase mRNA.

Cell Culture

Cultures of c^a/c^a and Black Silky chicken melanocytes were developed from embryonic neural crest cells according to the method of Giss *et al.* (1982) and Tobita *et al.* (1998). Briefly, a neural crest from a 22-somite stage embryo was dissected, and single cells were isolated with EDTA-trypsin[4] (Cat. No. 16170-086) digestion. The cells were cultured in F-12[4] (Cat. No. 21700-075) medium supplemented with 10% calf serum[4] (Cat. No. 610-5400AG) and 10 ng/mL endothelin-3[5] (Cat. No. E9139). After selective proliferation of melanocytes for 10 to 20 d, cells were collected using EDTA-trypsin[4].

RT-PCR and DNA Sequencing

Total RNA was isolated from the cultured melanocytes of albino and Black Silky chicken embryos by using an RNeasy Mini Kit[6] (Cat. No. 74103) according to the manufacturer instructions. Five micrograms of the obtained RNA was reverse transcribed with 200 units of Superscript™ II reverse transcriptase[4] (Cat. No. 18064-014) in a total volume of 20 μL containing the recommended buffer. For amplification of tyrosinase cDNA by RT-PCR, we designed two primers. The primers 5′-GTT CAT TGG AGC GGT CAG AG-3′ and 5′-TTG GAG GAC TGG AGC TGA TA-3′ were at the 5′- and 3′-untranslated regions, respectively, based on the sequence of White Leghorn chicken tyrosinase cDNA (Mochii *et al.*, 1992). The PCR was performed in a total volume of 96 μL comprising 0.5 μL of the sample solution containing RT product, 1.3 m*M* $MgCl_2$, 0.5 m*M* dNTP, 100 pmol each of the 3′ and 5′ primers, 5 units of *Pyrobest*™ DNA polymerase[7] (Cat. No. R005A) in the recommended buffer. The PCR was performed using 30 cycles under the following conditions: the first 10 cycles were at 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min; the second 10 cycles were at 94 C for 1 min, 52.5 C for 1 min, and 72 C for 1 min; the third 10 cycles were at 94 C for 1 min, 50 C for 1 min, and 72 C for 1 min followed by an elongation phase at 72 C. The resulting fragment was separated by electrophoresis using 1.5% L03 agarose gel[7] (Cat. No. 5003) and was approximately 1,800 bp, which corresponded to the expected size. This fragment was purified using a gel extraction kit[6] (Cat. No. 20021), and then DNA cycle sequenced according to the LI-COR DNA Sequencing System Protocol. The nucleotide alignment analysis was made with the GENETYX program.

RESULTS AND DISCUSSION

Melanocytes were cultured from albino and Black Silky (control) embryos. Cell proliferation and normal melanocyte morphology were observed in albino melanocytes but without pigmentation. The morphology of the melanocytes (data not shown) was similar to that reported in earlier studies (Akiyama *et al.*, 1994).

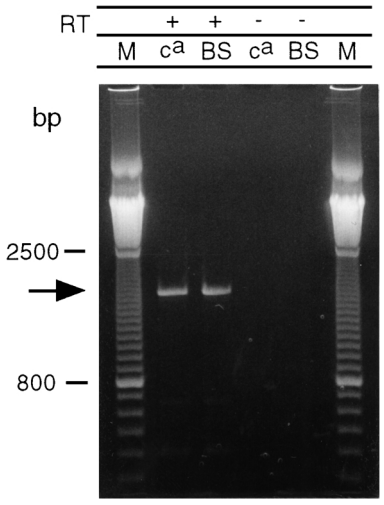

FIGURE 1. Polymerase chain reaction (PCR) amplification products of the tyrosinase cDNA of albino (c^a) and Black Silky (BS) chickens. Reverse transcriptase (RT)-PCR reaction (RT +) resulted in amplification of albino and Black Silky chickens tyrosinase cDNA of 1,800 bp (arrow), but PCR amplification without RT reaction (RT –) as a negative control did not produce any bands in the lanes of either bird. The molecular size marker (M) is 100-bp ladder.

[4]Life Technologies, Burlington, ON, Canada, L7P 1A1.
[5]Sigma Chemical Co., St. Louis, MO 63178-9916.
[6]QIAGEN Inc., 28159 Avenue Stanford, Santa Clarita CA 91355.
[7]Takara Shuzo Co. Ltd., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan.

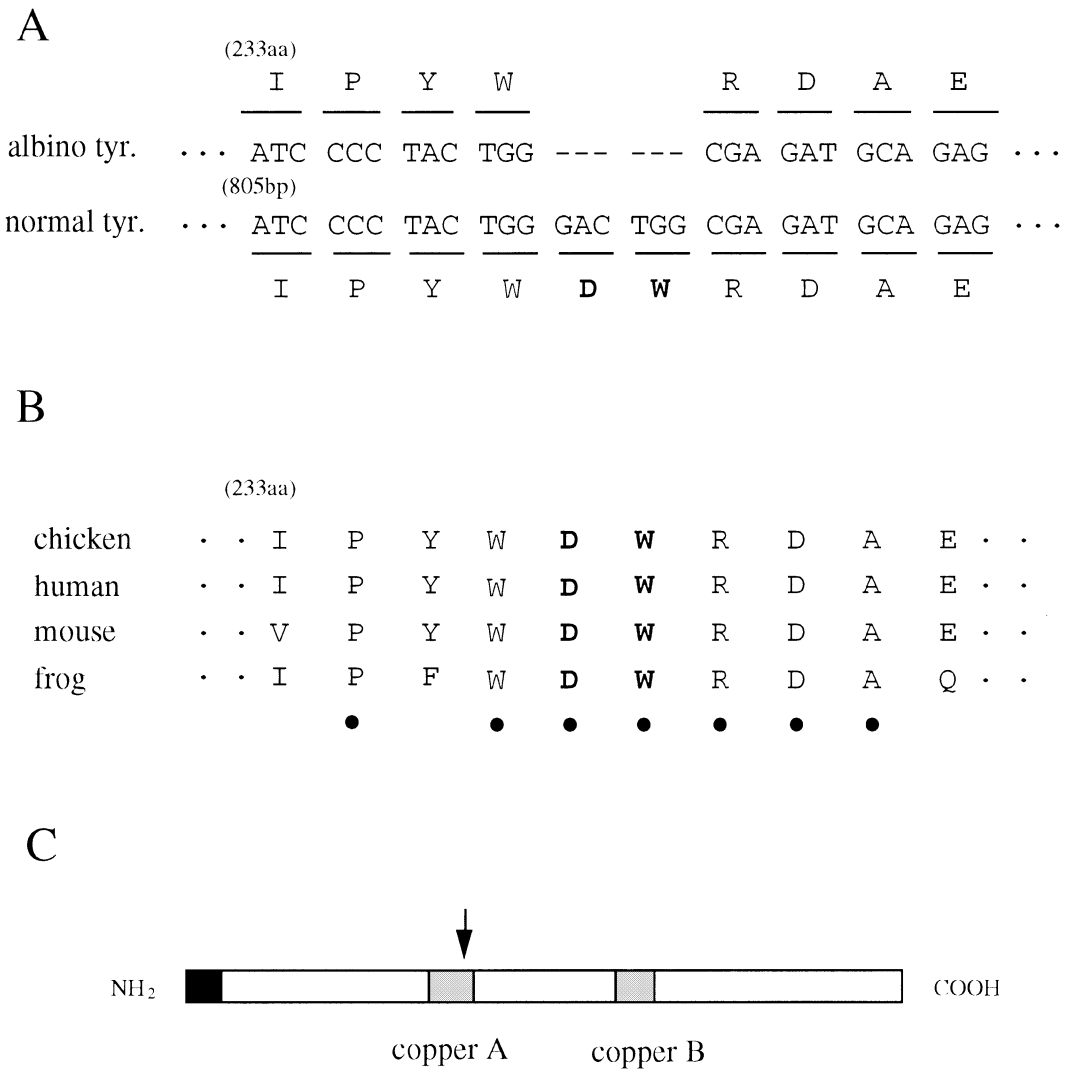

FIGURE 2. The DNA and deduced amino acid sequence of the mutated region (A). The sites of oligonucleotide deletion are indicated by small bars (–). The six-nucleotide deletion affected two codons. Deletion of two amino acids was found at 237, aspartic acid, and 238, tryptophan (indicated as bold letters). A comparison of amino acid sequences around the mutation site in chicken, human, mouse, and frog (B). GenBank accession numbers are D88394, Y00819, X12782, and D12514, respectively. Conserved amino acids are indicated as large dots. Mutation site in the tyrosinase gene (C). The c^a/c^a mutated site is indicated by an arrow. The locations of the two deduced copper-binding regions (copper A and B) (Shibahara *et al.*, 1988) are shown by shaded boxes. The closed box at the amino end shows the region of the cleaved signal peptide.

For amplification of the complete open reading frame (ORF) region of albino tyrosinase cDNA, we designed antisense and sense oligonucleotide primers (20mers) at the 5′- and 3′-untranslated regions of tyrosinase cDNA from White Leghorn chicken (Mochii *et al.*, 1992). Using these primers, we amplified the tyrosinase cDNA fragments containing the complete ORF of tyrosinase cDNA from the cultured melanocytes of c^a/c^a and control Black Silky. The PCR amplification produced a single band at approximately 1,800 bp from both samples on agarose gel electrophoresis (Figure 1). We could not detect any PCR products without RT or differences in the fragment size and amount of the PCR products between c^a/c^a and the control. These results showed that the albino chicken synthesizes tyrosinase mRNA and that the transcriptional regulatory region of the albino tyrosinase gene was probably functional.

We failed to obtain the tyrosinase-specific mRNA from tissues such as brain and liver from both lines of chickens (data not shown). Thus, the amount of tyrosinase mRNA in these tissues may be small. We recognized that the expression of the mutant tyrosinase mRNA in melanocytes was higher than that in other tissues such as muscle, brain, and liver from adult albino chickens (data not shown). Therefore, tyrosinase-specific mRNA in albino chickens was also controlled in a tissue-specific manner as normal tyrosinase mRNA from normally pigmented chickens.

By using direct sequencing of the PCR products, we found three mismatched sites in the DNA sequence of c^a/c^a tyrosinase cDNA when compared with the Black Silky or White Leghorn cDNA sequences. Two of these sites were nucleotide conversions, C757T and G849T, resulting in no alterations in amino acid sequence. The third

site was the six-nucleotide deletion (–ΔGACTGG) from 817 through 822 bp at codons 237 and 238, compared with the normal tyrosinase cDNA sequence as shown in Figure 2A. A six-nucleotide deletion in the tyrosinase structural gene has not been previously described in the tyrosinase gene from other albino vertebrates. This mutation deletes two amino acids in the tyrosinase protein: 237, aspartic acid, and 238, tryptophan.

We compared tyrosinase amino acid sequences around this six-nucleotide deletion site in related organisms (Figure 2B). Comparison of sequences showed that aspartic acid and tryptophan were conserved in a wide variety of organisms from amphibian to human, which indicated that these two amino acids had some structural or functional importance in tyrosinase. We investigated functional domains of the two amino acid deletion sites in the albino tyrosinase by using the functional domain map of human tyrosinase (Shibahara *et al.*, 1988). As shown in Figure 2C, the human tyrosinase enzyme contains two potential copper-binding regions, copper A and copper B, which may be important in the function of the enzyme (Kwon *et al.*, 1988). Our alignment analysis showed that the two amino acid deletion sites in c^a/c^a lay within the copper A domain of tyrosinase.

The deletion of a charged amino acid might alter the isoelectric point of the mutated tyrosinase protein. Oetting *et al.* (1985), however, indicated that the putative tyrosinase protein spots of c^a migrated the same as that from normally pigmented chickens using two-dimensional gel electrophoresis. Because native tyrosinase is an acidic protein with a pI of 4.0 to 4.5, one interpretation of their results is that such a change is too small to detect with two-dimensional gel electrophoresis. Alternatively, the hydrophobic nature of neighboring amino acids such as 233, isoleucine, and 234, proline, might internalize and hide the charge alteration. Because the tertiary structure of tyrosinase has not been ascertained yet, we can not make a definitive conclusion.

Boissy *et al.* (1987) indicated that antigenicity of tyrosinase from the same line of c^a chicken was clearly different from the normal tyrosinase protein by immunoprecipitation using antiserum against hamster tyrosinase. With reference to our current data, their report suggests that the missing two amino acids might alter the antigenicity of tyrosinase, indicating a change in the tertiary conformation of the protein.

Many different mutations in the copper A domain of human tyrosinase in albinos have been reported (King *et al.*, 1991; Oetting and King, 1993). These mutations were proposed either to disable the binding of copper to the enzyme or to interfere with the binding of molecular oxygen to the copper (Dressler and Dawson, 1960). We thus suggest that the mutation of c^a might affect the function of the copper-binding site A, resulting in nonfunctional tyrosinase in the albino chicken.

In conclusion, the c^a/c^a mutation in this line of albino chickens is a structural mutation of the tyrosinase gene in which there is a two amino acid deletion in the copper-binding region (copper A) of tyrosinase. There are multiple alleles at the *C* locus and other loci in the chicken varying from complete nonpigmentation to reduced pigmentation. These findings have not been analyzed regarding the molecular defect in the melanin biosynthesis pathway. Molecular analysis of mutations in these loci and in their protein products is important for the understanding of pigment production.

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